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Interaction between Human Natural Anti-α-Galactosyl Immunoglobulin G and Bacteria of the Human Flora†

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Anti-α-galactosyl immunoglobulin G (anti-Gal) is a natural antibody present in unusually high amounts in human sera. It constitutes as much as 1% of circulating immunoglobulin G in humans and displays a distinct specificity for the carbohydrate epitope galactosyl α(1→3) galactosyl (Galα1→3Gal). Recently, it has been suggested by various investigators that anti-Gal may be related to some autoimmune phenomena, since marked elevation of its titer was found in sera of patients with autoimmune thyroid disorders, rheumatoid arthritis, glomerulonephritis, and Chagas' disease. In view of the ubiquitous presence of anti-Gal in high titers in humans, throughout life, we hypothesized that, analogous with synthesis of anti-blood group antibodies against bacterial antigens, bacteria within normal intestinal flora may provide constant antigenic stimulation for the synthesis of anti-Gal. This hypothesis would imply that anti-Gal may bind to a variety of bacterial strains of human flora. In the present study, the interaction between affinity chromatography-purified anti-Gal and various bacterial strains was studied. By the use of a direct immunostaining assay and an enzyme-linked immunosorbent assay, anti-Gal was found to interact with a variety of *Escherichia coli*, *Klebsiella*, and *Salmonella* strains, some of which were isolates from normal stool. Furthermore, the anti-Gal-binding sites in some strains were found to be present on the carbohydrate portion of bacterial lipopolysaccharides. It is thus suggested that Galα1→3Gal epitopes in the outer membranes of normal flora enterobacteria may provide a continuous source for antigenic stimulation. Since there is no immune tolerance to the Galα1→3Gal carbohydrate structure in humans, anti-Gal seems to be constantly produced in response to these enterobacteria. In addition, bacteria which express Galα→3Gal epitopes and which may adhere to various cells mediated binding of anti-Gal to human cell lines. These findings raise the possibility that anti-Gal may damage normal human tissues via inflammatory processes facilitated by bacterial Galα1→3Gal epitopes.

Normal serum agglutinins, lysins, and complement-fixing antibodies for bacteria and erythrocytes were among the first observations in immunology (see reference 47). It has been argued by Wiener (47) that natural antibodies such as blood group hemagglutinins "are most likely immune antibodies of heterogenetic microbial origin." As a result of the understanding of the carbohydrate structure of blood group antigens (28, 46), it became apparent that natural anti-blood group antibodies interact with a variety of carbohydrate structures which are shared between bacteria and some human blood group antigens (see reference 37). Thus, many bacterial strains, including isolates from stool specimens, were found able to inhibit anti-blood group A or B agglutinins (24, 39). The hypothesis that anti-blood group antibodies are produced as a result of constant immunization against intestinal flora was further supported by studies of Springer and Horton (38) on the immune reaction to *Escherichia coli* O86, a gram-negative bacterium which possesses high blood group B and faint blood group A activity in vitro. These researchers found that humans of all ages exceeding 1 week may form increased amounts of anti-blood group A and B antibodies in response to ingestion or inhalation of *E. coli* O86.

We have recently (15–21; U. Galili, Transfusion Med. Rev., in press) described a human natural polyclonal anti-α-galactosyl immunoglobulin G(IgG) antibody designated anti-Gal which constitutes 1% of the total serum IgG in all individuals independent of blood type and which appears to participate in the destruction of normal senescent and some pathologic erythrocytes. Detailed analysis of anti-Gal carbohydrate specificity revealed that this antibody, when isolated from B or AB blood type serum, interacts specifically with galactosyl α(1→3) galactosyl (Galα1→3Gal) residues of various glycolipid molecules (15, 20). In individuals of A or O blood type, affinity chromatography-purified anti-Gal was found to contain antibody clones which, in addition to Galα1→3Gal specificity, may interact with Galα1→3(Fucα1→2)Gal, which is the blood type B antigenic epitope (15; Galili, in press). In fact, most of the so-called anti-blood group B reactivity could be attributed to anti-Gal antibody clones capable of interacting with both Galα1→3Gal and Galα1→3(Fucα1→2)Gal antigenic epitopes (15; Galili, in press). The presence in normal serum of high titers of natural antibodies recognizing Galα1→3Gal residues has also been demonstrated by other groups (1, 5, 9, 11, 30, 33, 41, 42). In recent studies, anti-Gal was demonstrated to be the natural human antibody which interacts with mouse laminin, and its titer was found to be elevated in patients with Chagas' disease and American cutaneous leishmaniasis (1, 42). Severalfold increases in anti-Gal titers were also observed in patients with Graves' disease and other autoimmune thyroid disorders (11), in some patients with rheumatoid arthritis

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(33), and in Henoch-Schönlein patients (9). These studies suggest that in humans, anti-Gal is related by an unknown mechanism to some autoimmune phenomena. In addition, anti-Gal was found to display a unique evolutionary distribution in primates (17). Anti-Gal is present in Old World monkeys and apes in titers comparable to those in humans, but is absent in New World monkeys. Conversely, the Galα1→3Gal epitope is abundantly expressed on erythrocytes of New World monkeys and many nonprimate mammals, but its expression is diminished on cells from Old World monkeys, apes, and humans. Other investigators reported on the presence of this epitope on cell surface glycoproteins and secreted glycoproteins in various mammals (5, 7, 8, 36, 40, 42, 44) but not in humans (36).

We hypothesized that, analogous with the findings on the natural anti-blood group antibodies, constant production of anti-Gal antibodies in humans throughout life may result from an ongoing antigenic stimulation by intestinal bacterial flora expressing Galα1→3Gal epitopes. Indeed, lipopolysaccharides (LPS) from certain bacterial strains were found, by chemical analysis, to contain the Galα1→3Gal epitope. Lüderitz et al. (32) proposed that the O-specific side chain of *Salmonella milwaukee* LPS has a repeating pentasaccharide unit of Galα1→3Galβ1→3GalNAc1→3GlcNAc1→4Fuc. Repeating units of Galα1→3Gal on *Salmonella* LPS has been demonstrated in more recent studies by using specific enzymatic degradations and nuclear magnetic resonance analysis (14, 27). Repeating units containing Galα1→3Gal have also been found in *Klebsiella* species (2, 6), and units containing galactosyls with an α1→3 linkage to a variety of carbohydrates have been described in LPS of many *E. coli* strains (26).

The purpose of the present study was to analyze the immunologic expression of the Galα1→3Gal epitopes on bacteria of normal flora by studying the interaction of intact live or fixed bacteria and of bacterial LPS with the natural anti-Gal. Demonstration of such interaction would support the hypothesis that bacteria of normal flora may provide the antigenic stimulus for anti-Gal production.

MATERIALS AND METHODS

Bacterial strains. This study employed bacterial strains isolated from human stool and strains isolated from the blood of patients at the Beth Israel Hospital, Boston, Mass., and characterized at the Center for Immunochemistry, Veterans Administration Hospital, San Francisco, Calif. The normal stool isolates include *E. coli* 15293 and *Klebsiella* strains 6613 and C5. The sepsis isolates include *Klebsiella* strains 18022, 18033, and 18052 and *Serratia marcescens* 18021. These strains are numbered according to an internal master list. The following heat-killed *Salmonella* strains were received from O. Westphal of the Max Planck Institute, Freiburg, Federal Republic of Germany: *S. minnesota* 1127, *S. typhimurium* 1591, and *S. milwaukee* 1139. *E. coli* O86 was received from G. Springer, Northwestern University, Evanston, Ill. *E. coli* K92 and *Klebsiella* strain 15314 were provided by Frits and Ida Ørskov, International Escherichia and Klebsiella Centre, Statens Seruminstitut, Copenhagen, Denmark.

Isolation of anti-Gal from normal AB plasma. Anti-Gal was isolated by affinity chromatography from the plasma of healthy individuals with blood type AB. Batches of 50 ml of heat-inactivated AB plasma (pooled from four donors) were loaded onto a 20-ml affinity chromatography SYNSORB 90 column with the oligosaccharide epitope Galα1→3Galβ1→4Glc (Chembiomed, Edmonton, Alberta, Canada). After extensive washing, the column-bound antibodies were eluted with 0.5 M melibiose. The carbohydrate was removed by repeated dialysis, and the purified anti-Gal was concentrated to 100 μg/ml, a concentration similar to that found in the blood. (Anti-Gal may also be isolated from AB sera by affinity chromatography on melibiose [α-galactosyl-glucoside]–Sepharose [5, 18, 21].)

Binding of anti-Gal to various bacterial strains. Anti-Gal interaction with bacteria was analyzed by several approaches, including immunostaining of live bacteria in colonies, enzyme-linked immunosorbent assay (ELISA) with fixed bacteria, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of bacterial LPS, blotting, and immunostaining of the blotted molecules with anti-Gal.

(i) Immunostaining of live bacteria grown in colonies. Bacterial colonies were grown on nutrient agar plates. Sterile nitrocellulose paper was applied onto the colonies and then gently lifted with colonies transferred to it. The paper was incubated for 30 min in a 1% casein solution in 10 mM Tris hydrochloride–150 mM NaCl–5 mM $MgCl_2$–3 mM NaN_3 (pH 7.4) for blocking and then in an anti-Gal solution (20 μg/ml) for 1 h. At the end of incubation with anti-Gal, the paper was washed with phosphate-buffered saline and incubated in a solution of alkaline phosphatase-conjugated goat anti-human IgG antibodies (Vector Laboratories, Burlingame, Calif.) for 1 h. After additional washing, a naphthol-phosphate substrate (20 mg/ml) and fast red solution (20 mg/ml) (Sigma Chemical Co., St. Louis, Mo.) were added in Tris buffer (pH 8.0) and the resulting development of pink color was followed.

(ii) ELISA with bacterial suspensions. This ELISA was performed with suspensions of fixed bacteria rather than with bacteria fixed to plates, in order to use precise concentrations of bacteria, when anti-Gal binding was compared in different strains. Various bacterial strains were fixed for 20 min with 0.5% glutaraldehyde. After reactive residues were blocked with 0.15 M glycine and 1% bovine serum albumin, the bacteria were brought to a concentration of 10^9 bacteria per ml. Samples of 50 μl were incubated in V-shaped microtiter wells with equal volumes of anti-Gal dilutions for 2 h at 24°C. At the end of incubation, the bacteria were washed five times with phosphate-buffered saline by centrifugation for 10 min at 1,000 × *g* the supernatant was removed, and the bacteria were resuspended in 200 μl of phosphate-buffered saline. The bacteria were then incubated with biotinylated goat anti-human IgG for 1 h (Vector Laboratories), washed, and incubated for 1 h with avidin-alkaline phosphatase (Vector Laboratories). After five washes, the bacteria were incubated for 30 min in 100 μl of the alkaline phosphatase substrate (Sigma 104-105), and the plates were then spun. The supernatants were transferred to new wells and scored for A_{405}. Bacteria undergoing the same procedure but not incubated with anti-Gal served as a control. Binding of IgG to Fc receptors was excluded, since the background control in all strains studied was low (<0.1 optical density unit). In bacteria with documented Fc receptors such as *Staphylococcus aureus*, the biotinylated anti-human IgG antibody bound to the bacteria. This resulted in a background level of >1.5 optical density units.

To demonstrate carbohydrate specificity of the bacterial-bound anti-Gal, we fixed bacteria with 0.5% glutaraldehyde to the bottom of flat microtiter wells. After being blocked with glycine and bovine serum albumin, the fixed bacteria were incubated with anti-Gal in the presence of various carbohydrates. The binding of anti-Gal was measured by

ELISA as described above. All ELISAs were performed in duplicate.

(iii) ELISA with bacterial sonicates adhering to human cells. The possible capability of bacterial fragments to adhere to human cells and subsequently cause the binding of anti-Gal to these cells was studied. Two bacterial strains, *E. coli* O86 and *Klebsiella* strain 18022, were brought in minimal essential medium with 10% fetal calf serum to a concentration of 10^9 bacteria per ml and fragmented by sonication for 30 min in a sonicating bath. The clear suspension of the bacterial sonicates indicated the extensive fragmentation. The sonicated bacterial suspensions were incubated with monolayers of normal human fibroblasts and of HeLa cells in 96-well plates for 18 h at 37°C. The plates were then extensively washed with minimal essential medium to remove nonadherent bacterial fragments. Adherent bacterial fragments were fixed with 0.5% glutaraldehyde (20 min at 24°C). After glutaraldehyde-reactive groups were blocked with glycine and bovine serum albumin solution, the cell monolayers were subjected to anti-Gal binding ELISA as described above. Endogenous alkaline phosphatase within the cell line monolayers was blocked by Levamisole (Vector Laboratories). The assay was performed in duplicate.

PAGE and Immunoblot Analysis of LPS. Separation of LPS samples through polyacrylamide gels was performed by the method of Laemmli (31) with minor modifications as previously described (34). Either purified LPS or LPS in proteinase K-whole-cell lysates was used as a sample for gels. Proteinase K was obtained from Sigma. The LPS samples were diluted to 50 μg/ml in a buffer consisting of 2% SDS in 60 mM Tris hydrochloride–1 mM EDTA–3.5% β-mercaptoethanol–2% bromophenol blue (pH 6.8) (sample buffer) and heated at 100°C for 5 min. Proteinase K-whole-cell lysate LPS samples were prepared in the sample buffer as previously described (23). The LPS samples were applied to duplicate slab gels and electrophoresed at 25 mA per slab for 5 h. The LPS on one slab was stained with silver (43), and the samples on the second slab were electroblotted onto nitrocellulose paper by applying 30 V for 5 to 18 h. The blotted paper was incubated in a 1% casein buffer for 1 h and reacted for 2 h with anti-Gal diluted in this buffer to a concentration of 10 μg/ml. The binding of anti-Gal was assessed by two methods. (i) The anti-Gal-treated nitrocellulose paper was washed extensively and incubated for 1 h with ^{125}I-labeled protein A. After being washed and dried, the paper was autoradiographed (Kodak X-OMat). Blotted preparations exposed only to ^{125}I-protein A were used as controls. (ii) After incubation with anti-Gal, the nitrocellulose paper was washed and incubated for 1 h with alkaline phosphatase-conjugated goat anti-human IgG antibody. A naphthol-phosphate substrate and fast red solution were then added, and the development of a pink color was assessed visually.

RESULTS

Anti-Gal binding to live bacteria in colonies. The binding of anti-Gal to bacteria could be demonstrated with bacteria grown on agar in colonies and transferred to nitrocellulose paper. Anti-Gal readily bound to *Klebsiella* strain 18033 (Fig. 1a) and to *E. coli* O86 (Fig. 1c). Anti-Gal binding resulting in lower intensity of immunostaining was observed with other *Klebsiella* and *E. coli* strains (data not shown). The specificity of antibody binding was demonstrated by the lack of observed color reaction when only secondary antibody and alkaline phosphatase reagent were applied onto the nitrocellulose paper with the blotted colonies (Fig. 1b and d).

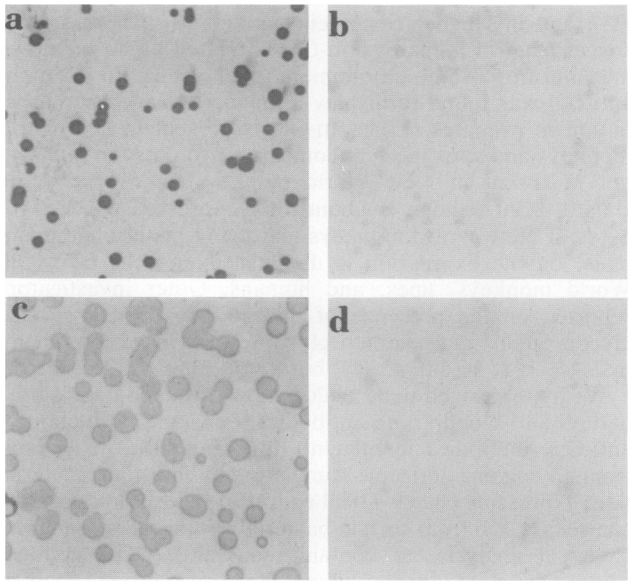

FIG. 1. Binding of anti-Gal to bacterial colonies transferred onto nitrocellulose paper. (a) Anti-Gal binding to *K. pneumonia* 18033 colonies. (b) Control of secondary antibody (goat anti-human IgG antibodies conjugated with alkaline phosphatase) with *K. pneumonia* 18033 colonies. (c) Anti-Gal binding to *E. coli* O86. (d) Control of secondary antibody with *E. coli* O86.

Interaction of anti-Gal with different bacterial strains of a given genus in ELISA. For comparison of the expression of Galα1→3Gal epitopes on various strains within a given genus, the binding of anti-Gal in various dilutions to 5×10^7 bacteria per well was assessed by ELISA. Binding of anti-Gal to *S. minnesota* was detectable at an antibody dilution of 1:320 (Fig. 2A). However, the binding to *S. typhimurium* yielded an enzyme reaction in the ELISA at an anti-Gal dilution of 1:80, and with *S. milwaukee* a reaction was seen at only 1:20. Within the *Klebsiella* genus (Fig. 2B), anti-Gal bound to strain 18033 much better than to strain C5, and no binding was detected with *Klebsiella* strains 18022 and 18052. A similar phenomenon was observed with the different *E. coli* strains, among which *E. coli* O86 showed higher affinity to anti-Gal than *E. coli* 15293 and almost no interaction was observed with *E. coli* K92 (Fig. 2C).

The α-galactosyl specificity of anti-Gal binding to bacteria could be demonstrated by inhibition of the binding with α-galactosyl-containing carbohydrates. A representative example is shown in Fig. 3. Anti-Gal binding to *Klebsiella* strain 18033 was greatly reduced by α-methylgalactoside and melibiose (α-galactosyl-glucoside) at anti-Gal titers of 1:5 and 1:20 and completely abolished at a titer of 1:80. In contrast, β-methylgalactoside and lactose (β-galactosyl-glucoside) had almost no inhibitory effect on anti-Gal binding to *Klebsiella* strains. The oligosaccharide Galα1→3Gal was not assayed for inhibition owing to lack of sufficient amounts of this compound.

Interaction of anti-Gal with carbohydrate structures on bacterial LPS. Galactosyl residues with α anomeric linkages are a common component in the LPS of members of the family *Enterobacteriaceae* (2, 6, 14, 22, 26, 32). It was therefore of interest to study the binding of anti-Gal to LPS of various bacteria. Because of the repeating nature of LPS carbohydrate units, finding an interaction of anti-Gal with LPS would suggest that this bacterial wall component is a powerful immunogen for anti-Gal production. LPS was

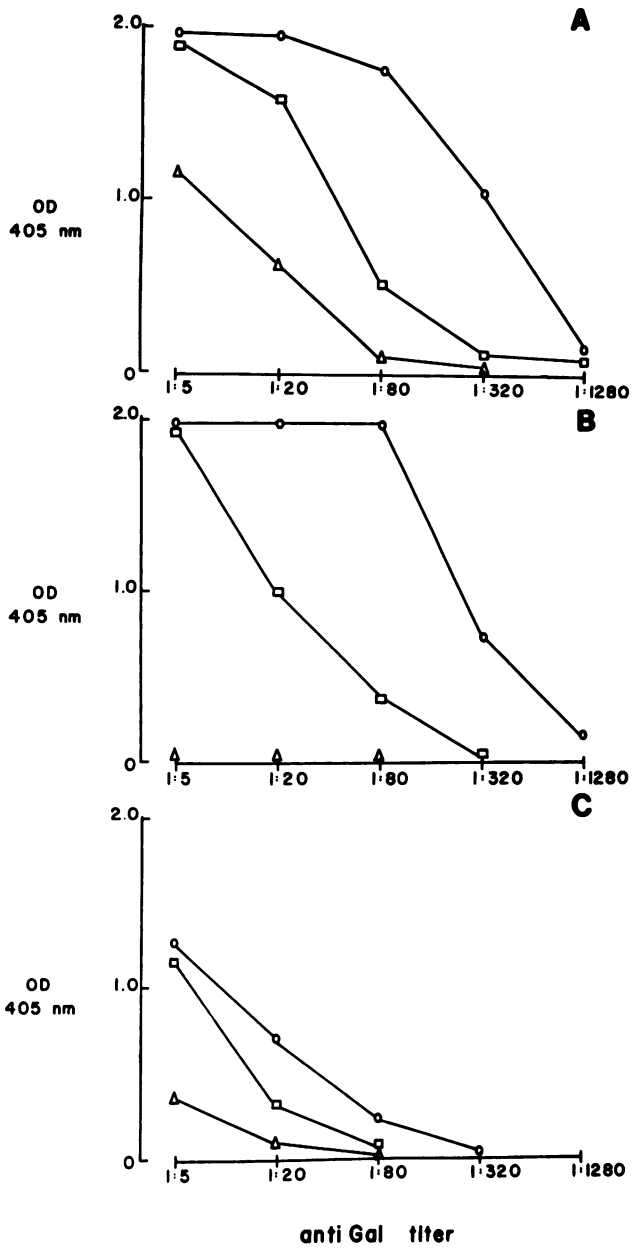

FIG. 2. Binding of anti-Gal to different bacterial strains as assessed by ELISA with 5×10^7 bacteria per well. (A) *S. minnesota* (○); *S. typhimurium* (□); *S. milwaukee* (△). (B) *Klebsiella* strain 18033 (○); *Klebsiella* strain C5 (□); *Klebsiella* strains 18022 and 18052 (△). (C) *E. coli* O86 (○); *E. coli* 15293 (□); *E. coli* K92 (△). OD, Optical density.

prepared from a variety of bacterial strains, separated by SDS-PAGE, blotted to nitrocellulose, and immunostained with anti-Gal.

The highly sensitive silver staining technique shown in Fig. 4 revealed that in all strains, discrete bands arranged in a pattern characteristic for carbohydrate molecules cumulatively increased in size by a defined increment, i.e., a repeating oligosaccharide unit (22, 23, 35). Anti-Gal immunostaining of blotted bacterial LPS with ^{125}I-protein A (Fig. 4) demonstrated the binding of this antibody to LPS molecules of *Klebsiella pneumoniae* 18022, *S. minnesota* 1127, and *E. coli* 15293. The diffuse pattern of LPS from *E. coli* is

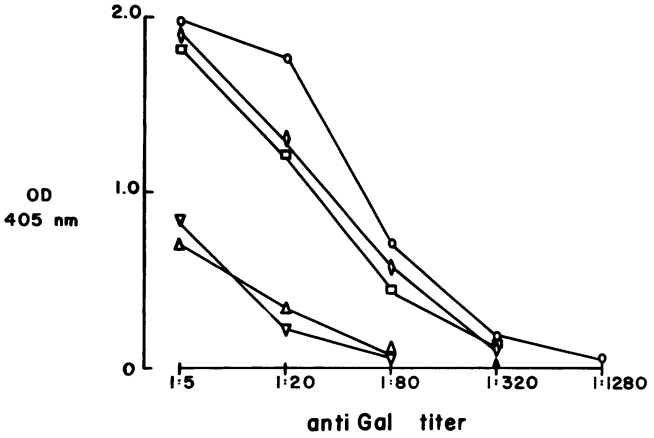

FIG. 3. Carbohydrate inhibition of anti-Gal binding to *K. pneumoniae* 18033. Anti-Gal was incubated in various dilutions with bacteria and in the presence of different carbohydrates (100 mM). The antibody binding was assessed thereafter by ELISA. Carbohydrates used were melibiose (△); lactose (□); α-methylgalactoside (▽); and β-methylgalactoside (◇). ○, PBS control. OD, Optical density.

due to large amounts of the antigenic material and the proximity of the bands (Fig. 4, lane F). No binding was observed in any of the LPS preparations to the lower-molecular-weight molecules which probably correspond to the core components (22, 35). The specificity of anti-Gal binding was demonstrated by the lack of binding of the antibody to the LPS of *Klebsiella* strain 6613 and by the lack

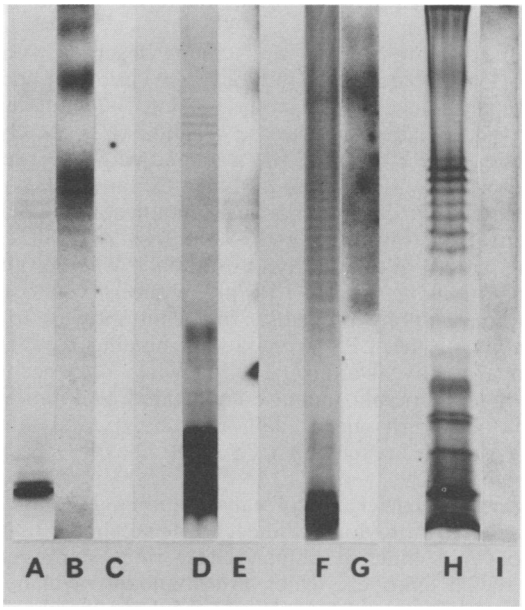

FIG. 4. Binding of anti-Gal to LPS separated by SDS-PAGE. Lanes: A, *K. pneumoniae* 18022 LPS, silver stain; B, *K. pneumoniae* 18022 LPS, immunoblot with anti-Gal; C, *K. pneumoniae* 18022 LPS, immunoblot without anti-Gal but only exposed to ^{125}I-protein A; D, *S. minnesota* 1127 proteinase K lysate, silver stain; E, *S. minnesota* 1127 proteinase K lysate, immunoblot with anti-Gal; F, *E. coli* 15293 proteinase K lysate, silver stain; G, *E. coli* 15293 proteinase K lysate, immunoblot with anti-Gal; H, *Klebsiella* strain 6613 LPS, silver stain; I, *Klebsiella* strain 6613 LPS, immunoblot with anti-Gal.

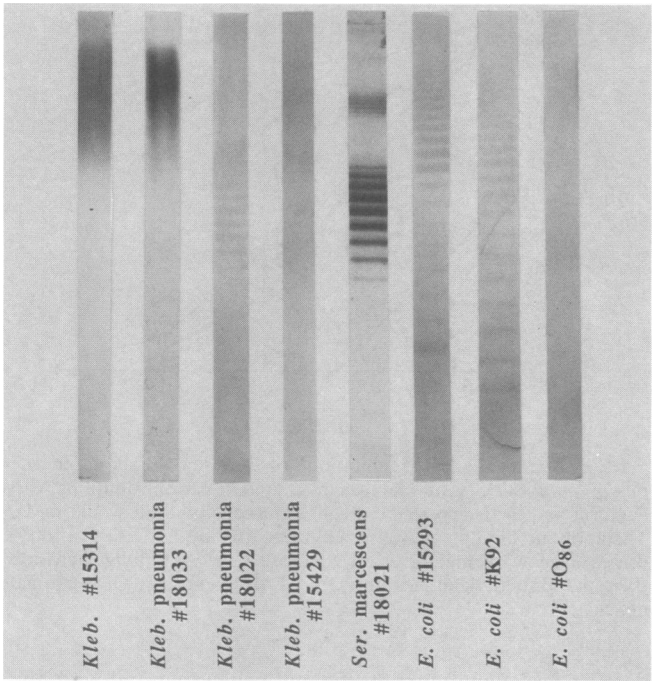

FIG. 5. Binding of anti-Gal to LPS from various bacteria separated by SDS-PAGE, as assessed by immunostaining with alkaline phosphatase-conjugated secondary antibody. No immunostaining was observed with only secondary antibody.

of labeling of LPS of *K. pneumoniae* 18022 when the blot was exposed to ^{125}I-protein A without initial exposure to the anti-Gal. Similarly, no direct ^{125}I-protein A binding was observed with LPS of the other bacterial strains studied (data not shown).

The fuzzy pattern of immunostaining observed with ^{125}I-protein A prompted us to study a larger variety of bacterial LPS preparations using the method of anti-Gal immunostaining by the alkaline phosphatase reaction (Fig. 5). By this technique, anti-Gal binding was detected with LPS preparations from *Klebsiella* strains 15314, 18022, and 18033 and *E. coli* K92 and 15293. The strongest immunostaining reaction was repeatedly observed with *S. marcescens* 18021. The staining pattern did not differ substantially when proteinase K preparations or purified LPS preparations were used for the chromatography procedure (data not shown). In addition, none of the LPS preparations binding the anti-Gal yielded a positive staining reaction when only secondary antibody (alkaline phosphatase-conjugated goat anti-human IgG antibody) was used (data not shown). No anti-Gal binding was observed with LPS from *E. coli* O86 and *K. pneumonia* 15429.

Fragmented bacteria may cause binding of anti-Gal to human cells. In previous studies, we have shown that anti-Gal does not bind to human cells, since expression of Galα1→3Gal epitopes, which are abundant in many nonprimate mammals and New World monkeys, has been evolutionarily suppressed in Old World primates and humans (17). It was of interest to determine whether bacterial fragments from strains expressing Galα1→3Gal epitopes may bind to human cells and cause the subsequent binding of anti-Gal to these cells. Such a finding may be of substantial significance since it would imply that under certain inflammatory conditions, bacteria immunizing humans to produce anti-Gal also may cause anti-Gal-mediated destruction of human tissues to which they adhere.

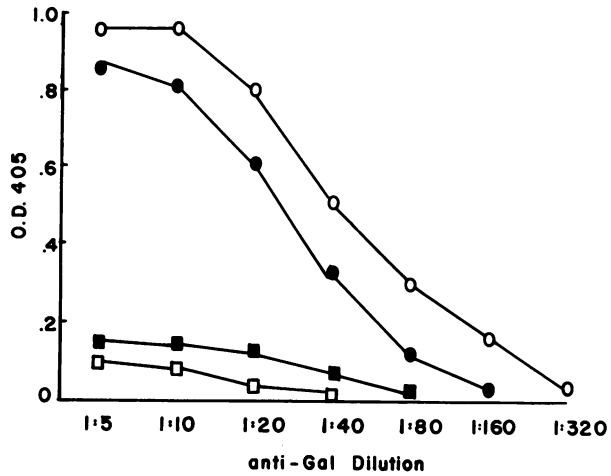

FIG. 6. Binding of anti-Gal to human fibroblasts (○, □) and HeLa cells (●, ■) after their exposure to fragmented bacterial suspensions of *E. coli* O86 (○, ●) or *K. pneumoniae* 18022 (□, ■). Antibody binding was assessed by ELISA in duplicate. OD 405, Optical density at 405 nm.

This possibility was studied with two bacterial strains demonstrated above to express Galα1→3Gal epitopes. *E. coli* O86 expressed this carbohydrate epitope on the surface and *K. pneumoniae* 18022 expressed it on the LPS molecules. After fragmentation of the bacteria by sonication, fibroblast and HeLa cells were incubated with the sonicates and the binding of anti-Gal to the cells was then assessed by ELISA. Anti-Gal did not bind to monolayers of fixed human fibroblast or HeLa cells (data not shown). Incubation of the two cell lines with a suspension of fragmented *K. pneumonia* 18022 also did not result in significant binding of anti-Gal (Fig. 6). However, after exposure of both cell lines to fragmented *E. coli* O86, substantial binding of anti-Gal was observed even when the antibody was used at a 1:40 dilution of the antibody concentration in normal sera (Fig. 6). It is not clear as yet whether the inability of anti-Gal to bind to the cells in monolayers preexposed to *K. pneumoniae* fragments is due to lack of adhesion of fragments to cells or to inaccessibility of Galα1→3Gal epitopes on this bacterium to anti-Gal binding.

DISCUSSION

The interaction of anti-Gal with various bacteria as observed in this study suggests that Galα1→3Gal epitopes are expressed in a variety of bacterial strains including gram-negative strains present in normal human intestinal flora. To establish this point, we studied anti-Gal binding to different bacterial strains in a number of antibody binding assays. In some bacteria, such as *E. coli* O86 and *Klebsiella* strain 18033, this epitope was readily expressed on the cell wall, as indicated by binding of anti-Gal to the live and glutaraldehyde-fixed bacteria. The α-galactosyl specificity of the interaction between anti-Gal and the bacteria was demonstrated by specific inhibition of binding with α-galactosyl-containing carbohydrates. A similar carbohydrate inhibitory pattern of anti-Gal binding to Galα1→3Gal epitopes was previously observed when Galα1→3Gal expression was studied on various erythrocytes (16, 17, 20, 21) and on mouse laminin (5, 42).

ELISAs with controlled concentrations of fixed bacteria suspensions indicated that the number of Galα1→3Gal resi-

dues expressed on the bacterial wall differed in various strains of a given genus.

Binding of anti-Gal to *S. minnesota* was observed at dilutions at which no anti-Gal binding was detectable on *S. milwaukee*. Similarly, one *Klebsiella* strain (18033) bound anti-Gal at a 1:320 dilution, whereas another strain (18052) did not reveal antibody binding, even at a 1:5 dilution of anti-Gal.

In an attempt to determine the site of the bacterial wall carrying the Galα1→3Gal epitope, we screened LPS preparations from various bacteria for binding of anti-Gal. In some bacteria, the LPS molecules separated by SDS-PAGE and blotted on nitrocellulose paper readily bound the antibody. These included LPS preparations from *Klebsiella* strains 18022 and 18033, *S. minnesota* 1127, *E. coli* 15293 and K92, and *S. marcescens* 18021. The multiple-band pattern of immunostaining in the form of a ladder observed both with ^{125}I-protein A and with alkaline phosphatase may reflect a defined repeating oligosaccharide increment in the LPS molecules (22, 35). The fuzziness of the lines in some of the preparations may be caused by limited diffusion in the gels before blotting. This binding of anti-Gal to LPS molecules of a number of bacteria suggests that the Galα1→3Gal structure is a common carbohydrate epitope on bacterial LPS. These data support previous observations, which suggested the occurrence of Galα1→3Gal epitopes as part of the carbohydrate moiety of LPS in various bacterial strains (2, 6, 14, 27, 32).

It is of interest to note that whereas live or fixed *E. coli* O86 bacteria bound anti-Gal, no binding of the antibody was observed with the LPS preparation of this bacterium. This suggests that the Galα1→3Gal epitope in *E. coli* O86 is expressed on the capsule or glycoprotein portion of the bacterial wall, rather than on the LPS molecules. Conversely, no binding of anti-Gal was observed with the fixed *Klebsiella* strain 18022, whereas the LPS molecules bound the antibody. These findings suggest that in this bacterium, the Galα1→3Gal epitope is under the capsule in a state which is not accessible to the antibody. Since this strain was a blood isolate, the inaccessibility of the epitope to anti-Gal may explain why such a bacterium can survive in the blood and evade the detrimental effect of anti-Gal. Further studies are needed to determine whether modulation of Galα1→3Gal epitopes is a mechanism used by bacteria with this structure for evading anti-Gal within the circulation.

The demonstration of the Galα1→3Gal epitope in a wide variety of bacterial strains suggests that such bacteria within the normal intestinal flora provide antigenic stimulation throughout life for the observed constant production throughout life of large amounts of anti-Gal (21). This assumption is further supported by observations on the marked increase in anti-Gal titer subsequent to infection with protozoa expressing Galα1→3Gal epitopes such as *Trypanosoma cruzi* in Chagas' disease patients and *Leishmania mexicana* in patients with American cutaneous leishmaniasis (1, 42). These findings indicate that there are lymphoid clones producing anti-Gal capable of responding to exogenous Galα1→3Gal antigenic stimuli.

Our previous studies indicated that anti-Gal binds in situ to a cryptic Galα1→3Gal epitope exposed de novo on the small (<1%) normal senescent erythrocyte population and labels these cells for phagocytosis by reticuloendothelial macrophages (16, 18, 19, 21). In view of these findings, it could be argued that such senescent erythrocytes, rather than bacteria, may serve as the source for antigenic stimulation for anti-Gal production. This is unlikely, since in patients with β-thalassemia or sickle cell anemia, in whom the proportion of erythrocytes binding anti-Gal is much higher than that in normal individuals (16, 19, 21), no significant elevation in anti-Gal titer was observed.

Our assumption that anti-Gal is produced as a result of exposure to bacterial carbohydrate antigens is also indirectly supported by studies of Springer and Horton (38), who reported on the elevation of anti-blood group B antibody titer subsequent to ingestion or inhalation of killed *E. coli* O86 bacteria. Our present studies demonstrated the binding of anti-Gal to *E. coli* O86. In previous studies (15; Galili, in press), we have shown that in blood group A and O individuals, most of the anti-B antibodies (>85%) are in fact anti-Gal antibodies capable of binding to both Galα1→3Gal residues and Galα1→3(Fucα1→2)Gal residues (i.e., blood group B antigen). These antibodies, designated anti-Gal B, are absent from blood group AB and B individuals because of immune tolerance. In view of these studies, the measured elevation of anti-B antibodies observed by Springer and Horton subsequent to feeding with *E. coli* O86 (38) is likely to have resulted from immunization toward the Galα1→3Gal epitopes on this bacterium and elevation of titer of anti-Gal B as well as anti-Gal antibodies.

In addition to its expression on the bacterial wall, our previous studies have indicated that the Galα1→3Gal epitope is also abundantly expressed on erythrocytes of many mammalian species including New World monkeys but that it is not detected on erythrocytes of Old World monkeys and anthropoid apes (17). In humans, this epitope seems to be present on erythrocytes in a cryptic form and is exposed upon aging of erythrocytes in the circulation or as a result of proteolytic removal of surface glycoproteins (16, 18, 19, 21). Other investigators have demonstrated binding of anti-Gal to trypsin-treated human thyroid cells, implying presence of cryptic Galα1→3Gal structures on other human tissues in addition to erythrocytes (11). This finding suggests that, as a result of antigenic sharing between cryptic epitopes on human tissues and immunogenic glycosidic structures on the bacterial wall, anti-Gal plays an important role in the initiation of autoimmune processes in tissues where such cryptic structures are exposed due to pathologic processes. Further studies are needed to determine whether the elevation of anti-Gal titers observed in patients with autoimmune thyroid disorders (11) or Henoch-Schönlein purpura (9) and in rheumatoid arthritis patients with renal injury (33) is related to such putative autoimmune processes.

The interaction between bacterial wall components and anti-Gal demonstrated in the present study may contribute to inflammatory processes which, in addition to reacting against invading bacteria, may result in the excessive damage of autologous tissues in humans. This assumption is supported by findings that fragmented *E. coli* O86 bacteria adhere to normal fibroblasts and HeLa cells and subsequently mediate the binding of anti-Gal to the surface of these cells. An in vivo inflammatory process involving bacteria with similar characteristics may result in adherence of bacterial fragments with Galα1→3Gal epitopes to surrounding cells. This would result in anti-Gal-mediated reaction against such cells. It would be hard to demonstrate such a putative process in vivo. However, the finding of bacteria with Galα1→3Gal epitopes within inflammatory sites with excessive damage to healthy tissue may suggest the occurrence of this mechanism.

An additional hypothetical contribution of bacteria to anti-Gal-mediated autoimmunity in humans stems from the unique structure of the Galα1→3Gal nonreducing residue in

mammalian cells. The Galα1→3Gal epitope is usually synthesized on the lactosamine portion of glycoconjugates of many mammals as Galα1→3Galβ1→4GlcNAc-R (7, 8, 25, 36, 40, 44 45). Since the lactosamine structure Galβ1→4Glc NAc-R is also common on human cells (12, 13), the diminished expression of Galα1→3Gal epitopes on human cells seems to be related to an evolutionary suppression of the gene encoding the α1→3 galactosyltransferase (17, 36). This enzyme, which is highly active in various mammals (3, 4, 45), links the terminal α-galactosyl residue to lactosamine structures to form the Galα1→3Gal epitope. It is likely that a similar enzymatic activity should be present within bacterial strains which produce Galα1→3Gal epitopes, and indeed such an enzyme has been isolated from *S. typhimurium* (10). The possible coexistence of lactosamine glycoconjugates on human cells and enterobacteria which are capable of producing α1→3 galactosyltransferase prompts us to speculate that under certain conditions, Galα1→3Gal structures on human cells might be generated by the bacterial enzyme, using human cell surface lactosamine chains as acceptor molecules. Since such Galα1→3Gal epitopes would be recognizable by 1% of circulating antibodies in humans (i.e., anti-Gal), this might represent the initiation of an autoimmune process in tissues expressing such a neoantigen. A similar mechanism was proposed to mediate autoimmune phenomena observed in Chagas' disease, in which the infectious agent *T. cruzi* was also found to express Galα1→3Gal epitopes (42). We are currently analyzing the α1→3 galactosyltransferase activity in bacterial strains found in the present study to interact with anti-Gal to determine whether the bacterial enzyme can use cellular lactosamine-containing glycoconjugates as acceptors for the formation of Galα1→3Gal epitopes.

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